Title: Biofilms formed by isolates from recurrent vulvovaginal candidiasis patients are heterogeneous and insensitive to fluconazole

Leighann Sherry[1], Ryan Kean[1,2], Emily McKloud[3], Lindsay E. O'Donnell[1], Rebecca Metcalfe[4], Brian L. Jones[5] and Gordon Ramage*[1]

[1]Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK, [2]Institute of Healthcare Policy and Practice, University of West of Scotland, Paisley, UK, [3]Department of Health & Life Sciences, Glasgow Caledonian University, UK, [4]Sandyford Sexual Health Service, NHS Greater Glasgow & Clyde, UK, [5]Microbiology Department, Glasgow Royal Infirmary, UK

Running title: Vaginal biofilms are resistant to fluconazole

Key words: *Candida*, biofilm, vulvovaginal candidiasis, fluconazole

WORD COUNT:

Text: 950

References: 26

Figures: 2

Tables: 1

*Corresponding Author: Gordon Ramage, Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, University of Glasgow, 378 Sauchiehall Street, Glasgow, G2 3JZ, UK. Phone: +44(0)141 211 9752. e-mail: gordon.ramage@glasgow.ac.uk

Abstract (75 word limit)

Vulvovaginal candidiasis (VVC) is a global health problem affecting ~75% of women at least once in their lifetime. Here we examined the epidemiology of VVC from a patient cohort to identify the causative organisms associated with VVC. Biofilm forming capacity and antifungal sensitivity profiles were also assessed. We report a shifting prevalence of *Candida* species with heterogeneous biofilm forming capacity, both of which are associated with altered antifungal drug sensitivity.

Fungal infections play a surprisingly substantial, yet unrecognised, health burden on the global population (1). Vulvovaginal candidiasis (VVC) is one example of these, where it is estimated to be the most common fungal infection in a number of countries worldwide (2-4). Approximately 138 million women worldwide complain of >4 episodes of VVC per year due to treatment failure, clinically defined as recurrent VVC (RVVC) (5-7). These unresolved infections not only have a high impact on the quality of life of these women, but can also lead to further health complications (8). *Candida albicans* is historically reported as the predominant organism isolated from VVC, accounting for over 90% of infections (9, 10). However, evidence of a dynamic shift in yeast epidemiology has been demonstrated through an increasing prevalence of non-*C. albicans* species (NCAS), which accounts for 11-80% of infections, depending on geographical location (11). Nevertheless, *C. albicans,* a well-characterised biofilm-forming organism, remains a prominent pathogen in this disease. Resistance to antifungal therapy as a result of biofilm formation is a likely contributor to failed treatment. While it is widely accepted that biofilms contribute to the pathogenesis of bacterial vaginosis (BV) (12, 13), their role in VVC remains contested despite the overwhelming evidence to suggest otherwise (14-16).

An anonymised series of high vaginal swabs (HVS, [n=300]) obtained from women attending GP and referral clinics in the NHS Greater Glasgow & Clyde area, for at least the second time throughout April 2016 (17). These women were symptomatic at the time of sampling, with the causative organism identified using matrix-assisted laser desorption/ionisation-time of flight

(MALDI-TOF), with *Escherichia coli* used pre and post yeast sampling to ensure accuracy of testing.

Seventy one percent (n=212) identified as *C. albicans*, followed by 15% (n=47) *C. glabrata*, 6% (n=17) *C. dubliniensis*, 3% (n=10) *C. parapsilosis* (Figure 1). The remaining 5% of isolates included *C. tropicalis*, *C. lusitaniae* and *C. guillermondii*. These data are line with recent epidemiological patterns showing a shift in NCAS within VVC (11). However, a caveat of our study is the limitation of a single geographical location, which may influence the species distribution. Future studies should include various institutes globally in order to fully assess the shift in VVC epidemiology.

To determine the biofilm forming capability of these isolates, all VVC strains (n=300) were standardised to 1×10^6 cells/mL in RPMI-1640 and grown as biofilms in 96 well plates for 24 h. Biofilms were washed with PBS and biomass assessed using the crystal violet (cv) assay (18). Here we have shown that vaginal isolates were able to form differential biofilms, regardless of species (Figure 2). *C. albicans* displayed the greatest heterogeneity with regards to biofilm biomass, with isolates ranging from OD_{570nm} 0.008 to 1.478, with a mean of 0.416. The second most prevalent species, *C. glabrata*, had significantly lower biomass than *C. albicans* ($p<0.05$) and *C. dubliniensis* ($p<0.01$), with a mean OD_{570nm} 0.271. This apparent biofilm heterogeneity may contribute to the management of VVC infections, as these communities are known to be notoriously recalcitrant to antifungal therapy, and biofilm heterogeneity has been shown to correlate with *in vitro* antifungal therapy (18).

Planktonic and biofilm antifungal susceptibility testing was carried out as described previously to determine the minimum inhibitory concentrations (MICs) (19). Briefly, cells were standardised in RPMI-1640 before being treated with fluconazole (FLZ) (Sigma, Dorset, UK) for 24 h, at a range of concentrations (0.0625 to 32 mg/L). Planktonic MIC's (pMIC) were determined as the lowest concentration able to completely inhibit growth visually. Sessile MIC's (sMIC) were performed on 24 h preformed biofilms, with sMIC recorded at 50% inhibition using an XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide) metabolic reduction assay (20). Here we have shown FLZ, the first line antifungal used to treat VVC, was ineffective against most isolates, with planktonic MIC's ranging from <0.0625 to >32 mg/L (Table 1). Specifically, the $pMIC_{50}$ for FLZ was 4 mg/L, for *C. albicans*, *C. glabrata* and *C. dubliniensis*, though for biofilms this was >32 mg/L. When planktonic cells were stratified based on *C. albicans* and NCAS it was shown that 41% and 26% of the isolates were insensitive to FLZ at >32 mg/L, respectively. Whereas for sessile cells, this rose to 51% and 56% of the isolates, respectively. Interestingly, similar susceptibility profiles were observed for *C. albicans* and *C. glabrata*, despite *C. glabrata* known to be a low biofilm former (21). This reduced sensitivity in *C. glabrata* can be associated with its intrinsic resistance to fluconazole, due to the overexpression of multidrug transporters (22).

VVC is not a reportable disease, making epidemiological studies difficult. However, this study provides a snapshot of the species identified within a VVC population, demonstrating that NCAS are responsible for an increasing number of these infections. This corresponds with previous studies reporting an on-going dynamic shift in yeast epidemiology (23, 24), potentially driven by

inappropriate use of over-the-counter azoles (10). Irrespective, *C. albicans* remained the most dominant species in this study, which questions why a high number of isolates displayed reduced susceptibility to FLZ. We demonstrated the ability of these clinical isolates to form heterogeneous biofilms, and the presence of these communities in VVC may explain why *C. albicans* infections remain unresponsive to FLZ therapy; an antifungal highly ineffective against *C. albicans* biofilms (25). We cannot discount the potential for heteroresistance phenotypes within these populations (26). The contribution of biofilms to VVC pathogenesis remains poorly understood, though many researchers are beginning to consider them important determinants of disease (14, 15), further emphasising the need for research in this field. Collectively, the data from this investigation highlights the necessity for careful consideration of the causative organism in VVC, the biofilm phenotype and its accentuated antifungal sensitivity profiles, all of which may improve antifungal treatment in this area.

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Figure 1: Distribution of organism isolated from VVC patients. Three hundred VVC isolates were identified using MALDI-TOF, with yeast species proportionally represented.

Figure 2: VVC isolates display varied biofilm formation. Three hundred VVC isolates were screened for biofilm formation using a biomass stain, as described in the methods. Each isolate was tested in quadruplicate, with the mean represented. Statistical analysis was carried out using a one-way ANOVA (* $p<0.05$, ** $p<0.01$).

Table 1: Susceptibility profile of *Candida* vaginal isolates to fluconazole

Fluconazole Minimum Inhibitory Concentration (mg/L)										
n = 300	***C. albicans* (n=212)**		***C. glabrata* (n=47)**		***C. dubliniensis* (n=17)**		***C. parapsilosis* (n=10)**		**Others (n=14)**	
	PMIC*	**SMIC****	**PMIC**	**SMIC**	**PMIC**	**SMIC**	**PMIC**	**SMIC**	**PMIC**	**SMIC**
Range	0.0625 – >32	0.125 - >32	<0.0625 – >32	0.5 - >32	0.125 – >32	0.125 - >32	1 - >32	1 - >32	0.0625 – >32	1 - >32
MIC_{50}	4	>32	4	>32	4	>32	1	4	1	>32
MIC_{90}	>32	>32	>32	>32	>32	>32	16	>32	>32	>32

*PMIC – Planktonic minimum inhibitory concentration, **SMIC – sessile minimum inhibitory concentration

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Range	0.0625 – >32	0.125 - >32	<0.0625 – >32	0.5 - >32	0.125 – >32	0.125 - >32	1 - >32	1 - >32	0.0625 – >32	1 - >32
MIC_{50}	4	>32	4	>32	4	>32	1	4	1	>32
MIC_{90}	>32	>32	>32	>32	>32	>32	16	>32	>32	>32

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Figure 1

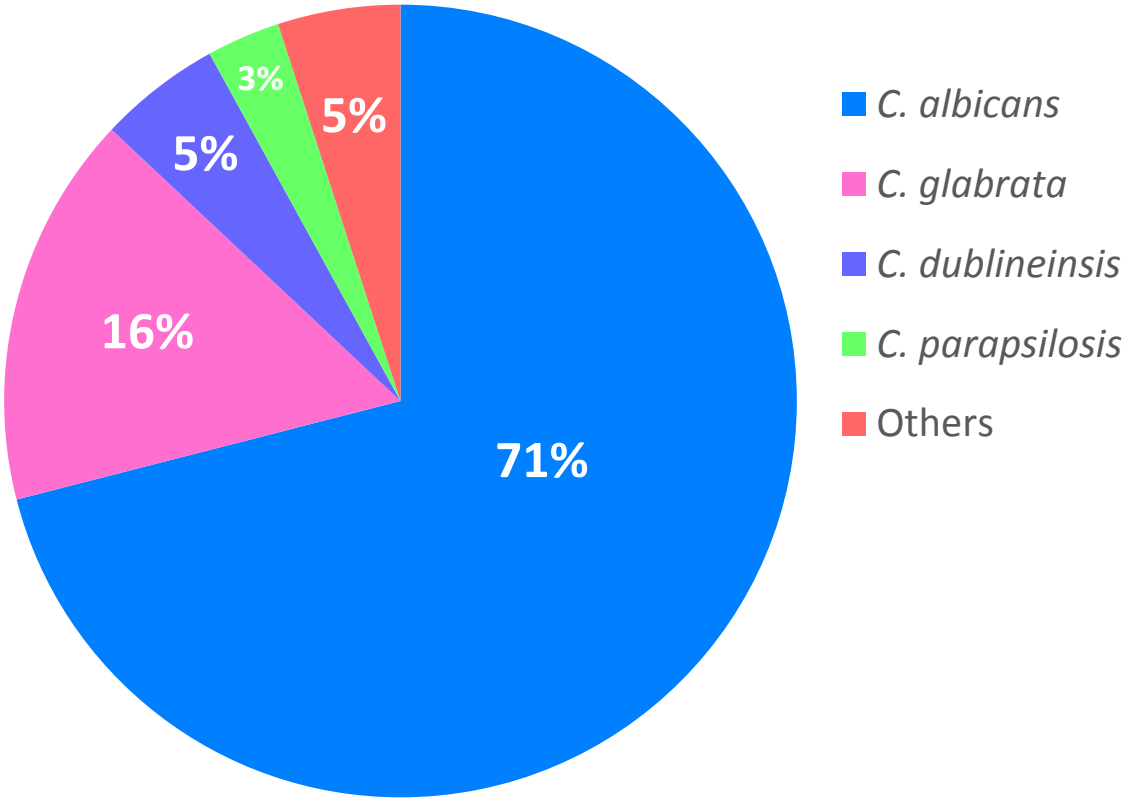
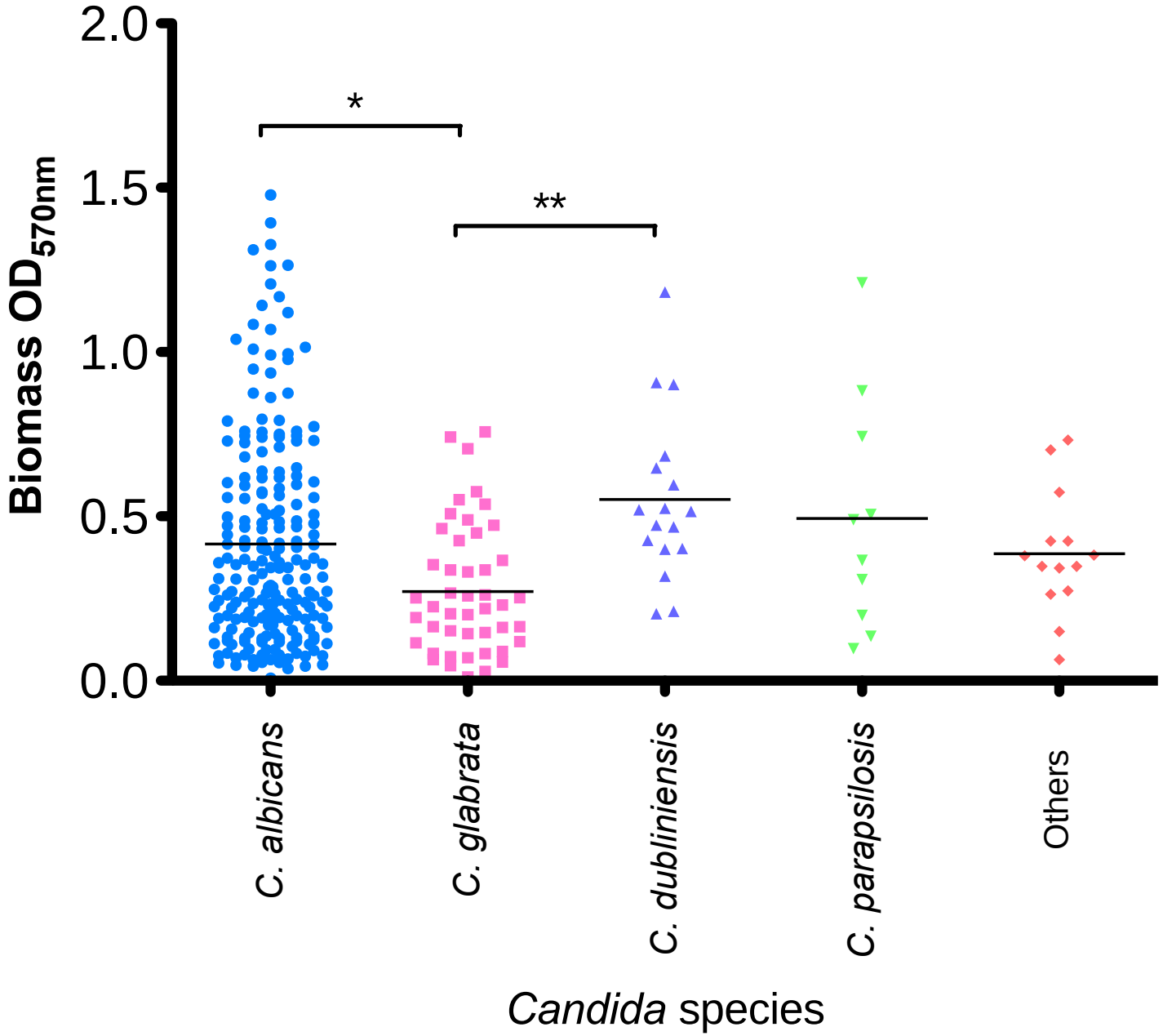

71%
16%
5%
3%
5%
C. albicans
C. glabrata
C. dublineinsis
C. parapsilosis
Others

Figure 2


2.0
1.5
1.0
0.5
0.0
Biomass OD570nm
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C. albicans
C. glabrata
C. dubliniensis
C. parapsilosis
Others
Candida species